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㉑ Culture medium for CHO-cells and adapted CHO-cells.

㉑ A biochemically defined culture medium for culturing engineered Chinese hamster ovary (CHO) cell lines, which is essentially free from protein, lipid and carbohydrate isolated from an animal source, comprising water, an osmolality regulator, a buffer, an energy source, amino acids including L-glutamine, an inorganic or recombinant iron source, and a synthetic or recombinant growth factor, and optionally non-ferrous metal ions vitamins and cofactors; also cells adapted to grow in such a culture medium.

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The present invention relates to a biochemically defined culture medium for culturing Chinese hamster ovary (CHO) cell lines and cells adapted to grow in the culture medium.

Chinese hamster ovary cells (CHO) were first cultured by Puck (J.Exp.Med. 108, 945, 1958) from a biopsy of an ovary from a female Chinese hamster. From these original cells various workers have developed a number of sub-lines with various deficiencies, one of which, CHO-K1, is proline-requiring and is diploid for the dihydrofolate reductase (dhfr) gene. From this cell line a dhfr⁻ CHO cell line (CHO DUK B11) was developed (PNAS 77, 1980, 4216-4220) which is characterised by the loss of dhfr function as a consequence of a mutation in one dhfr gene and the subsequent loss of the other gene. These cells are functionally dhfr⁻. Other CHO DUK sub-lines have been derived which are also phenotypically dhfr⁻. CHO cells which are dhfr⁻ cannot grow without nucleotide precursors such as thymidine, hypoxanthine, or the equivalent nucleosides.

Various proteins have been expressed in such CHO cells including the *E.coli* XGPRT gene (J.Mol.App.Gen. 1981, 1, 165-175), human tissue-type plasminogen activator (Mol. & Cell Biol. 5, 1750-1759, 1985), human immune (γ) interferon (PNAS 80 pp 4654-4658), and human beta interferon (Molecular and Cellular Biology 4, 166-172, 1984). A dhfr⁻ CHO cell line is transfected with a product gene and a dhfr gene which enables selection of CHO cell transformants of the dhfr⁺ phenotype. Selection is carried out by culturing the colonies in media devoid of thymidine and hypoxanthine, the absence of which prevents untransformed cells from growing. The transformants usually express low levels of the product gene by virtue of co-integration of both transfected genes. The expression levels for the product gene may be increased by amplification using methotrexate. This drug is a direct inhibitor of the dhfr enzyme and allows isolation of resistant colonies which have amplified their dhfr gene copy number sufficiently to survive under these conditions. Since the dhfr and product genes are usually closely linked in the original transformants, there is normally concomitant amplification resulting in increased expression of the desired product gene.

A different system of selection and amplification is provided by the glutamine synthetase selectable marker (or GS system) which is described in WO87/04462. CHO cells which have been successfully transfected with the gene encoding the GS enzyme and the desired antibody gene can be selected by culturing colonies in media devoid of glutamine and amplifying by the addition of methionine sulfoximine (Msx) as described in PCT published application number WO87/04462.

Engineered CHO cells (those in which a CHO cell line is transfected with a product gene and a selectable marker gene) are routinely grown in culture media containing serum. (References: J.Mol.App.Gen. 1981, 1, 165-175; Mol. & Cell Biol. 5, 1750-1759, 1985; PNAS 80 pp 4654-4658; Molecular and Cellular Biology 4, 166-172, 1984). Fetal bovine serum (FBS) is probably the most extensively utilised serum for mammalian cell culture, although other mammalian sera are used. However, the use of serum poses a number of problems. Serum is an expensive commodity which is not readily available in amounts required for commercial production. It is also a biochemically undefined material. Serum is known to contain many major components including albumin and transferrin and also minor components many of which have not been fully identified nor their action determined, thus serum will differ from batch to batch possibly requiring testing to determine levels of the various components and their effect on the cells. Frequently, serum is contaminated with microorganisms such as viruses and mycoplasma many of which may be harmless but will represent an additional unknown factor. This problem has become more acute in recent years with the emergence of Bovine Spongiform Encephalopathy (BSE). Despite improvements in screening, regulatory authorities are likely to require the sourcing of bovine products from those areas which are free from (BSE) infections.

Furthermore, the presence of animal proteins in culture media can require lengthy purification procedures, in particular the presence of bovine antibodies in bovine serum albumin (BSA) makes purification of the desired antibodies expressed by the recombinant CHO cell line, extremely difficult. Removal of bovine antibody from the medium prior to use is possible but this and the additional product testing required, adds greatly to the overall cost of production of the product. Consequently, there has been much research into finding a culture medium devoid of animal components which will support cellular growth, especially of CHO cells. Unfortunately, the problems associated with the provision of such a medium are themselves numerous. CHO cells do not readily grow in serum-free conditions. In addition, the removal of serum may also remove those components that provide cell protection and detoxifying activity.

A culture medium which is serum-free but not free from animal components is described by Mendiaz *et al* (In Vitro Cellular & Development Biology Vol.22, No.2, 1986) for use in the culture of CHO K1 cells. The medium is a modification of the medium developed by Ham (Microbiology 53 1965 288-293) which is known as "Ham's F12". Other examples of media have been based on Ham's F12 medium for example as disclosed in EPA390327 and EP325190. These media contain transferrin as the serum substitute, but transferrin is derived from an animal source, so the resulting media do not overcome the contamination problems associated with the use of serum.

A further problem which arises with the use of serum-free media is that of supporting recombinant CHO

cells to enable growth and expression of product. Media based on Ham's F12 which are not supplemented with serum are generally not rich enough to support full growth or expression.

Engineered CHO cells are also difficult to grow in suspension. It is highly desirable to achieve growth in suspension when using the cells to express a product such as an antibody. For production of a biological protein on a commercial scale it is preferable to be able to support growth in fermenters which range from 1 litre glass vessels to multi-thousand litre stainless steel tanks. A suitable medium must be able to support the cells against sheer forces from blade impellers or turbines and from effects of sparging (ie: supplying air, oxygen and CO₂ in bubble form directly to the medium).

The present invention therefore provides a biochemically defined culture medium for culturing engineered CHO cells which is essentially free from protein, lipid and carbohydrate isolated from an animal source, comprising water, an osmolality regulator, a buffer, an energy source, amino acids including L-glutamine, an inorganic or recombinant iron source and a recombinant or synthetic growth factor and optionally non-ferrous metal ions, vitamins and cofactors.

The components of the medium are mostly inorganic, synthetic or recombinant and as such are not obtained directly from any animal source. Some components may be obtained from a plant or bacterial source. Recombinant components are prepared under highly pure conditions to minimise the risk of contamination from the parent tissue passing to the cells used to produce the components. Further purification steps may be employed to remove cell proteins. Thus, a medium which is essentially free from all protein, lipid and carbohydrate isolated from an animal source, can be achieved. The preferred culture medium of the invention contains no protein, lipid and carbohydrate isolated from an animal source.

It is advantageous to maintain osmolality in the range 200-350mOsm preferably in the range 290-350mOsm. Osmolality regulators are generally salts. Those which may be used in the medium include NaCl, KCl, KNO₃.

Buffers of use in the medium to maintain the pH in the range 6.5 - 7.5 most preferably around pH 7.0. Buffers of use in the medium include carbonates such as NaHCO₃; also chlorides, sulphates and phosphates such as CaCl₂·2H₂O, MgSO₄·7H₂O, NaH₂PO₄·2H₂O, or sodium pyruvate, such buffers are generally present in an amount 50-500 mg/litre. Other buffers, such as N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] otherwise known as HEPES and 3-[N-Morpholino]-propanesulphonic acid otherwise known as MOPS are generally present in an amount 1000-10,000 mg/litre.

The energy source of use in the medium is generally present in an amount 1000-10,000 mg/litre and is preferably a monosaccharide such as manose, fructose, galactose or maltose most preferably glucose, particularly D-glucose.

The non-ferrous metal ions optionally of use in the medium include magnesium, copper and zinc; also sodium, potassium and selenium. The ions are generally added to the medium in the form of salts such as chlorides and sulphates. The amounts are typically similar to those provided in the ISCOVES medium set out in Table 1 but clearly may be varied.

Vitamins and enzyme co-factor vitamins (co-factors) optionally of use in the medium include Vitamin B6 (pyridoxine), Vitamin B12 (cyanocobalamin) and Vitamin K (biotin) present in an amount 0.01 - 0.5 mg/litre; Vitamin C (ascorbic acid) present in an amount 10 - 30 mg/litre, Vitamin B2 (riboflavin) present in an amount 0.1 - 1.0 mg/litre and Vitamin B1 (thiamine), nicotinamide, Vitamin B5 (D calcium pantothenate), folic acid, L-inositol generally present in an amount 0.2 - 8.0 mg/litre.

It is preferable to include in the basal medium a lipid factor such as choline chloride, lipoic acid, oleic acid, phosphatidylcholine or methyl linoleate, generally in an amount 0.05 - 10 mg/litre. Compounds involved in lipid production for example alcoholamines such as ethanolamine may also be added.

It is preferable to include additional amino acids in the medium selected from:

	<u>Amino Acid</u>	<u>Preferred mg/litre</u>
	L-Alanine	20 - 50
5	L-Arginine (HCl)	50 - 100
	L-Asparagine (H ₂ O)	20 - 50
	L-Aspartic Acid	20 - 50
10	L-Cystine (disodium salt)	50 - 100
	L-Glutamic acid	50 - 100
	L-Glutamine	400 - 600
	Glycine	20 - 50
15	L-Histidine (HCl.H ₂ O)	30 - 60
	L-Isoleucine	50 - 150
	L-Leucine	50 - 150
20	L-Lysine (HCl)	100 - 200
	L-Methionine	20 - 50
	L-Phenylalanine	40 - 80
	L-Proline	30 - 60
25	L-Serine	30 - 60
	L-Threonine	50 - 120
	L-Tryptophan	10 - 20
30	L-Tyrosine (disodium salt)	50 - 120
	L-Valine	80 - 120

The bracketed forms are preferred.

35 The amino acids are preferably of synthetic origin. The amounts which are usually included vary for each amino acid but are generally in the range 10 - 150 mg/ml. However, L-glutamine is generally present at much higher concentration preferably in the range 400-600 mg/ml.

It may be advantageous to include in the medium a pH indicator for example Phenol red sodium salt for example at 5 - 50 mg/litre.

40 Medium A as set out in Table 1, is an example of a medium which provides the preferred quantities of water, osmolality regulator, buffer, energy source, amino acids, non-ferrous metal ions, vitamins and co-factors as a basis for a culture medium according to the invention. This medium does not contain any hypoxanthine or thymidine and is commercially available from GIBCO Ltd., Unit 4, Cowley Mill Td. Est., Uxbridge UB8 2YG. It is similar to a published culture medium (Iscoves and Melcher (1978) J.Exp.Med. 1. 47,923) but does not contain any

45 bovine serum albumin, pure human transferrin or soyabean lecithin.

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Tabl 1**Medium A (modification of Iscoves' DMEM lacking albumin, transferrin and insulin)**

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	Ingredient	mg/litre
10		
	L-Alanine	25.00
	L-Arginine HCl	84.00
15	L-Asparagine H ₂ O	28.40
	L-Aspartic Acid	30.00
	L-Cystine	70.00
20	L-Glutamic acid	75.00
	L-Glutamine	584.00
	Glycine	30.00
	L-Histidine HCl.H ₂ O	42.00
25	L-Isoleucine	105.00
	L-Leucine	105.00
	L-Lysine HCl	146.00
30	L-Methionine	30.00

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	L-Phenylalanine	66.00
	L-Proline	40.00
5	L-Serine	42.00
	L-Threonine	95.00
	L-Tryptophan	16.00
	L-Tyrosine disodium salt	104.20
10	L-Valine	94.00
	Biotin	0.013
	D.Calcium Pantothenate	4.00
15	Choline chloride	4.00
	Folic acid	4.00
	D-Inositol	7.20
	Nicotinamide	4.00
20	Pyridoxal HCl	4.00
	Riboflavin	0.40
	Thiamin HCl	4.00
25	Vitamin B 12	0.013
	CaCl ₂ ·2H ₂ O	219.00
	KCl	330.00
30	KNO ₃	0.076
	MgSO ₄ ·7H ₂ O	200.00
	NaCl	4505.00
	NaHCO ₃	3024.00
35	NaH ₂ PO ₄ ·2H ₂ O	141.30
	D-Glucose	4500.00
	HEPES	5958.00
40	Phenol red sodium salt	15.00
	Sodium pyruvate	110.00
	Sodium selenite	0.017

45 DMEM modification of Iscoves N and Melcher (1978), J.Exp.Med. 1, 47, 923.

It is preferable to add to the medium, selenium (optionally in the form of sodium selenite) generally in an amount 0.01 - 0.2 mg/litre or L-Ascorbic acid generally in an amount 20 - 50 mg/litre to help minimise the potential toxic effects of ferrous or ferric ions, and oxygen. Further use of chelating agents such as citrate or Ethylenediaminetetraacetic acid (EDTA) or a free radical scavenger such as α -Tocopherol (vitamin E) are advantageous in reducing free radical damage.

Antibiotics such as polymyxin, neomycin, penicillin or streptomycin may be added to the medium to prevent bacterial contamination. These are usually included in an amount 10,000 - 100,000 IU/litre

Growth factors which may be added to the basal medium are synthetic or recombinant and include insulin. Other factors such as platelet-derived growth factor (PDGF), thyroxine T₃, thrombin, interleukins such as IL2 and IL6, progesterone, hydrocortisone and vitamin E may be included. Folic acid, vitamin B6 and vitamin B12 which are involved in the folate pathway may be added to enhance the growth of cells.

The peptide hormone insulin (which in the present context includes analogues thereof such as Nucleon (TM of Eli Lilly) is advantageously obtained by recombinant DNA techniques but is not isolated from an animal

source. It is preferably added to the medium in an amount 5µg - 5mg/litre. Nucellin is the preferred form of insulin for use in the invention.

The non-animal derived iron source to supplement the medium, is preferably inorganic and present in an amount 0.25 - 5mg/litre. Examples include ferric and ferrous salts such as ferric citrate or ferrous sulphate. The chelated salts such as ferric citrate and ferric ammonium citrate are preferred. However, any iron source may be used which is not isolated from an animal source, for example, chemical iron chelators or recombinant protein iron carriers.

The concentration of ferric or ferrous ions should be carefully controlled as these may help generate superoxides and free radicals in the medium, which may damage not only the cells themselves, but medium components and the desired end product.

It is also preferable to add to the medium, a compound such as putrescine, advantageously as a salt such as HCl, which is known to play a role in maintaining the structure of the endoplasmic reticulum and to be required by certain CHO cell lines to support growth. Putrescine or a salt thereof is preferably added in an amount 0.01 - 1.0 mg/litre.

Serum-free media disclosed to date contain hypoxanthine or thymidine. This could bypass the selection pressure placed on the dhfr selection and amplification system as previously disclosed. The result may be loss of genetic material specifying the product and the dhfr genes. Therefore, in another aspect of the invention there is provided a culture medium for the growth of engineered dhfr⁻ CHO cells in accordance with the invention, essentially free from hypoxanthine and/or thymidine.

The culture medium of the present invention supports CHO cell growth and when supplemented with an appropriate agent such as methotrexate for the dhfr system usually in an amount 0.1 - 5.0 µM, (or MSX for the GS-system), allow full selection pressure to be exerted on the cells. It will be understood that hypoxanthine and thymidine at concentrations which are insufficient to bypass selection of the dhfr system may be present in the medium, but the presence of these two nucleotide precursors is not preferred for use with the present invention.

In large scale fermenters, mammalian cells are particularly susceptible to sheer forces arising from the sparging of the vessel with gases and the mixing with the impeller. To minimise the occurrence of cellular damage it is advantageous for the medium to contain a cell protectant such as polyethylene glycol, polyvinyl alcohols or pluronic polyols. Of these, Pluronic (TM of BASF Wyandotte Corp) polyol F68 is preferred since unlike polyvinyl alcohols this is a non-toxic substance and unlike polyethylene glycols does not interfere with downstream purification.

Further improvements in CHO cell growth may be obtained by supplementing the medium with a peptide digest, hydrolysates or extracts, such as Tryptone, casein hydrolysate, yeast extract, or preferably papain digested soya peptone. The preferred amounts are 1% - 0.025% w/v, most preferably 0.25% w/v.

The media of the invention for culturing recombinant CHO cells are capable of supporting the growth and secretion of product from such cells in suspension in small and large scale fermenters, static cultures and/or spinners. The culture medium according to the invention is also capable of supporting growth of cells at high cell density namely greater than 1×10^5 cells/ml up to or greater than 1.5×10^6 cells/ml and product secretion of 30 mg/l up to greater than 150mg/l. The medium according to the invention is also capable of supporting this growth and product secretion over multiple passages lasting up to or greater than 6 months.

The medium is preferred for the production of all types of antibodies natural and altered. The invention therefore includes production of human antibodies wherein the amino acid sequences of the heavy and light chains are homologous with those sequences of antibodies produced by human lymphocytes *in vivo* or *in vitro* by hybridomas. Also provided are hybrid antibodies in which the heavy and light chains are homologous to a natural antibody but are combined in a way that would not occur naturally. For example, a bispecific antibody has antigen binding sites specific to more than one antigen. The constant region of the antibody may relate to one or other of the antigen binding regions or may be from a further antibody. Altered antibodies, for example chimaeric antibodies have variable regions from one antibody and constant regions from another. Thus, chimaeric antibodies may be species/species chimaeras or class/class chimaeras. Such chimaeric antibodies may have one or more further modifications to improve antigen binding ability or to alter effector functioning. Humanised or CDR-grafted antibodies (EP 239400) are embraced within the invention, in particular Campath 1H (EP328404) (Campath is a TM of The Wellcome Foundation) also composite antibodies, wherein parts of the hypervariable regions in addition to the CDRs are transferred to the human framework. Additional amino acids in the framework or constant regions of such antibodies may be altered. The invention further includes the production of Fab fragments which are roughly equivalent to the Y branch portions of the heavy and light chains; this includes incomplete fragments or fragments including part of the Fc region.

In a further aspect of the invention there is provided an engineered CHO cell adapted to grow in a medium according to the invention. In particular a CHO cell engineered to express proteins such as tissue plasminogen

activator or antibodies as defined above. In particular the invention provides a dhfr- CHO cell line transfected with a gen encoding a biologically active protein and a dhfr selectable marker gene, adapted to grow in a culture medium according to the invention. The protein is preferably an antibody as defined above.

The ingredients of the culture medium may be added in any order but it is preferable to add the iron source and when used, tyrosin , last to avoid precipitation.

Accompanying Figures are for illustration only.

Figure 1 shows growth of C1H 3D11* 44 in WCM5 (protein-free medium) in a 1 litre fermenter measured as cell count/ml over 90 days.

Figure 2 shows antibody production from C1H 3D11* 44 cells in WCM5 in a 1 litre fermenter measured as micrograms of antibody/ml over 80 days.

Example 1.

Formulation for medium WCM4.

Medium A: (Iscoves modification of DMEM without BSA, transferrin and lecithin as set out in Table 1).

20	+	5 ml/litre	200mM L glutamine
	+	50 mg/litre	L proline
	+	50 mg/litre	L threonine
25	+	50 mg/litre	L methionine
	+	50 mg/litre	L cysteine
	+	50 mg/litre	L tyrosine
30	+	25 mg.litre	ascorbic acid
	+	0.062 mg.litre	vitamin B6
	+	1.36 mg.litre	vitamin B12
	+	0.2 mg/litre	lipoic acid
35	+	0.088 mg/litre	methyl linoleate
	+	1 μ M	methotrexate
	+	1 mg/litre	FeSO ₄
40	+	1 mg/litre	ZnSO ₄
	+	0.0025 mg/litre	CuSO ₄
	+	5 mg/litre	recombinant insulin (Nucellin)
45	+	50,000 Iu/litre	polymyxin
	+	20,000 Iu/litre	neomycin
	+	0.16 mg/litre	putrescine-2 HCL.

50 This medium does not contain hypoxanthine, thymidine or folic acid which can bypass methotrexate selection. The medium does contain glycine which cannot by itself bypass selection. Therefore, this medium maintains full selection for methotrexate resistance.

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Exempl 2.**Formulation for Medium WCM5**

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Medium A: (Iscoves modification of DMEM without BSA, transferrin or lecithin).

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+ 5 ml/litre 200mM L glutamine

+ 50 mg/litre L proline

+ 50 mg/litre L threonine

15

+ 50 mg/litre L methionine

+ 50 mg/litre L cysteine

+ 50 mg/litre L tyrosine

+ 25 mg/litre L ascorbic acid

20

+ 0.062 mg.litre Vitamin B6

+ 1.36 mg.litre Vitamin B12

+ 2 mg/litre Ferric citrate

25

+ 1 mg/litre Zinc sulphate

+ 0.0025 mg.lit Copper sulphate

+ 50,000 IU/litre Polymyxin

+ 20,000 IU/litre Neomycin

30

+ 3 µl/litre Ethanolamine

+ 0.16 mg/litre Putrescine

+ 5 mg/litre Recombinant Insulin (Nucellin)

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Example 3**Growth of and Production from C1H 3D11* 44 in WCM4**

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C1H 3D11* cells are genetically engineered CHO DUK B11 cells (Urlaub and Chasin (1980)) PNAS 77, 7 pp 4216-4220). CHO DUK B11 cells cannot produce dihydrofolate reductase (dhfr). These cells were engineered to produce a humanised IgG antibody, Campath 1H (Winter et al., Nature, 1988, 322, 323-327), using plasmid constructs to express heavy and light antibody chains and the mouse dhfr. Expression is amplified and maintained using the folate antagonist methotrexate. C1H 3D11* cells growing as a monolayer in Iscoves + 10% FBS Flow, non-essential amino acids, 10^{-6} M Methotrexate and antibiotics were approximately 90% confluent. These cells were removed from the plastic with trypsin/versene, washed in Iscoves medium without supplements, centrifuged and resuspended at 5×10^4 /ml in WCM4 medium + 0.25% peptone + 0.1% polyethylene glycol (PEG) 10,000 + 0.5% fetal bovine serum (FBS) without methotrexate (MTX). Three 25cm² flasks were set up with 10ml of cell suspension + hypoxanthine (H), thymidine (T) or HT. These flasks were incubated at 36.5°C in 5% CO₂ incubator.

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After six days, the flasks were pooled and added to an equal volume of WCM4 + MTX without peptone or PEG, and were transferred to a 75cm² flask.

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These cells were used to seed a 500ml Techner spinner, incubated at 36.5°C spinning at 40 rpm. Cells continued growing serum free for a period of over five months and although it was found that the cells needed a period of adaptation, the growth rate and viability steadily improved. The population doubling time was calculated to be 73.1 hours over approximately 7 weeks; this decreased to 47.4 hours over the subsequent 20 days then stabilised. Antibody secretion remained high at levels in excess of 60 µg/ml. It was determined that the gene copy number in these cells did not decrease according to band intensity using Northern blot analysis.

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In fermenters, these cells produced antibody in excess of 70µg/ml and regularly achieved levels of 100µg/ml or more. The cells are denoted C1H 3D11* 44.

Example 4

Growth and Production of C1H 3D11* 44 in WCM5 in a 1 litre fermenter.

C1H 3D11*44 cells from Example 3 which had been growing serum-free for over 2 months were transferred to a SGI 1 litre fermenter with a stainless steel angled paddle turning at 70rpm. The temperature was set at 37°C, dO₂ at 10% and pH control to 7-7.2. The fermenter was seeded on day 0 with 0.22 x 10⁶ cells/ml in WCM4 (Example 1) with 0.1% polyethylene glycol (PEG) 10,000 and 0.25% soy peptone, and was top gassed with O₂. The cells were routinely passaged using fresh medium and a split rate typically between 1 to 2 and 1 to 4.

On day 33 the top gassing was replaced with deep sparging which is can be expected to cause more physical damage to the cells.

On day 50 onwards WCM5 (Example 2) was used together with peptone and PEG instead of WCM4.

On day 53 the PEG was replaced with 0.1% pluronic F68. The resulting growth and antibody levels achieved are shown in the attached graphs (Figs 1 and 2), and demonstrate the capacity of the invention to allow protein-free production of antibody in excess of 100µg/ml in fermenters.

Example 5

Growth of CHO AJ19 MCB1 in WCM4 and compared to CHO AJ19 MCB1 grown in serum containing medium

Chinese hamster ovary cells CHO AJ19 MCB1, derived from CHO DUK cells, (Urlaub & Chasin PNAS, 77, 7, pp4216-4220, 1980), were genetically engineered to produce tPA under methotrexate selection. This cell line had been routinely grown in a fermenter as a suspension culture using normal growth medium consisting of RPMI 1640 medium (GIBCO), 2.5% acid hydrolysed adult bovine serum (Imperial), 0.5% Tryptone, 50IU/ml polymycin, 20IU/ml neomycin, 500nM methotrexate (MTX).

Medium WCM4 was formulated to which was added:-

46B 0.25%w/v N-Z Soy Peptone (Sigma P1265), 0.1%w/v Polyethylene glycol (PEG) 20,000 (Serva, Carbowax 20M), 1uM MTX.

46C 0.25%w/v Yeast extract (Sigma Y0500), 0.1%w/v PEG 20,000 1uM MTX. In this medium the Iscoves' in CM4 was replaced by RPMI 1640 medium (ICN FLOW).

46D 0.25%w/v Yeast extract, 0.1%w/v PEG 20,000, 1uM MTX.

46E 0.25%w/v Yeast extract, 0.1%w/v PEG 20,000, 0.25% Foetal bovine serum (Imperial), 1uM MTX.

The yeast extract, Peptone and PEG were made up as 10% w/v solutions with water (Wellcome media production unit) and filtered through a 0.2µm disposable filter (Gelman, Supor Vac), then diluted for use. The cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

Cells growing in normal growth medium were pelleted by centrifugation at 1200g +4°C for 5 minutes, were washed in RPMI 1640 without supplements and pelleted again. The cells were then resuspended at 10⁵ cell/ml in normal growth medium (46A) and the other media (46B, 46C, 46D or 46E). 24 well plates (Costar 16mm wells) were seeded with 1ml/well and incubated, at 37°C in an incubator containing 5% CO₂. On days 3, 4, 5 and 6 one well of each was counted using a haemocytometer and trypan blue exclusion. Two further wells of each were harvested, pooled and pelleted at 1200g +4°C 5 minutes. The supernatant was separated and stored at -20°C. These samples were subsequently assayed for tPA. On day 6 samples from 46A and 46D only were harvested.

RESULTS

tPA specific activities in various crude harvests

Crude material produced in the five different media were tested using a QA validated ELISA assay to measure the tPA antigen concentrations µg/ml using binding to a polyclonal antibody against tPA, and clot lysis assay to measure tPA activity in IU/ml. From these results (Table 2), the specific activities were calculated.

Table 2

5	EXPERIMENT	DAYS IN CULTURE	CELLCOUNT X10-5		MEAN tPA ACTIVITY IU/ml (n=3)	MEAN tPA CONTENT ug/ml (n=3)	SPECIFIC ACTIVITY MegIU/mg
			VIABLE	NONVIABLE			
10	46A	3	3.5	0.1	3051	10.51	0.290
	46A	4	3.7	0.3	4841	14.85	0.326
	46A	5	4.1	0.2	5306	15.52	0.335
15	46A	6	5.8	0.5	8235	23.22	0.355
	46B	3	5.2	0.1	2552	10.44	0.244
	46B	4	7.2	0.3	5310	18.58	0.286
20	46B	5	7.8	0.2	6230	22.19	0.281
	46C	3	3.8	0.2	2779	9.61	0.289
	46C	4	4.9	0.3	3536	16.54	0.214
	46C	5	5.6	0.3	4639	19.88	0.233
25	46D	3	7.5	0.2	4650	17.66	0.263
	46D	4	8.3	0.8	7369	25.99	0.285
	46D	5	7.4	1.0	7882	24.26	0.325
30	46D	6	6.1	2.0	8095	27.06	0.299
	46E	3	6.4	0.1	6262	23.85	0.263
35	46E	4	7.3	0.5	10180	29.70	0.343
	46E	5	6.1	1.3	9080	34.25	0.265

From the above table there was no change of the specific activity in the five different crudes. The yield of tPA from protein free medium B, C and D was nearly equal to the yield of tPA from standard growth medium in group A and E.

Example 6 Continuous growth of CHO AJ19 MCBI in WCM4

CHO AJ19 MCBI in WCM4 cells growing in normal growth medium were pelleted and washed as in Example 5 and were resuspended at 7×10^4 /ml in 500ml of medium 46B. These cells were transferred to a Techne spinner flask and incubated, as above, stirring at 40rpm. At various time intervals the cells were counted and subcultured using the same medium. A sample was taken for tPA assay and treated as in Example 5.

The specific activity of tPA in various cell subcultures

The specific activity of supernatants from different pass levels of cells grown in WCM4 with peptone and 0.1% PEG 20K were measured by a combination of ELISA and clot lysis assay. The specific activities of different cell passages are summarised in Table 3.

Table 3

DAYS	PASS	CELLCOUNT X10 ⁻⁵		SPLIT RATE	tPA present in supernatant		
		VIABLE	NONVIABLE		conc. ug/ml (n=3)	tPA ACTIVITY IU/ml (n=3)	SPECIFIC ACTIVITY Meg. U/mg
7	1	9.75	0.65	1-10	ND	ND	ND
10	2	4.95	0.01	1-5	ND	ND	ND
13	3	6.35	0.0	1-10	22.2	8865	0.399
16	4	3.8	0.0	1-10	7.25	1914	0.264
21	5	7.2	0.8	1-10	15.08	4331	0.287
24	6	4.1	0.3	1-10	8.28	2040	0.246
30	7	5.3	0.4	1-6	7.30	2052	0.281
34	8	5.2	0.32	-	13.65	3518	0.258
36	8	7.95	0.10	1-8	18.60	5327	0.286
37	8	ND	ND	-	20.68	5526	0.267
38	8		100%	-	19.10	5474	0.287
38	9	12.00	0.5	1-5	20.85	8348	0.400
43	10	5.5	0.12	1-5	7.38	1888	0.256
48	11	4.4	0.19	1-6	13.4	3143	0.235
12	Experiment terminated						

ND = not done.

Over a 48 day period, based on the above split rate, one cell could have divided to give 3.77×10^8 cells. This is equivalent to 31.8 population doublings with a doubling time of 36 hours.

The results of the experiments conducted in Examples 5 and 6 demonstrate that the serum free media of the present invention is capable of supporting cell growth and tPA yield comparable to that achieved in serum containing media.

Claims

1. A biochemically defined culture medium for culturing engineered CHO cells which is essentially free from protein, lipid and carbohydrate isolated from an animal source comprising water, an osmolality regulator, a buffer, an energy source, amino acids including L-glutamine, an inorganic or recombinant iron source, a recombinant or synthetic or growth factor, and optionally non-ferrous metal ions, vitamins and cofactors.
2. A medium as claimed in claim 1 wherein the osmolality regulator maintains the medium at 200 - 350 mOsm.
3. A medium as claimed in claim 1 wherein the buffer is capable of maintaining the medium in the pH range 6.5 - 7.5.
4. A medium as claimed in claim 1 wherein the energy source is present in an amount 1000 - 10,000 mg/litre.

5. A medium as claimed in claim 4 wherein the energy source is a monosaccharide.
6. A medium as claimed in claim 1 wherein the amino acids are one or more selected from:
 - L-Alanine
 - 5 L-Arginine
 - L-Asparagine
 - L-Aspartic Acid
 - L-Cystine
 - L-Glutamic acid
 - 10 Glycine
 - L-Histidine
 - L-Isoleucine
 - L-Leucine
 - L-Lysine
 - 15 L-Methionine
 - L-Phenylalanine
 - L-Proline
 - L-Serine
 - L-Threonine
 - 20 L-Tryptophan
 - L-Tyrosine
 - L-Valine
7. A medium as claimed in claim 1 wherein the L-glutamine is present in an amount 400 - 600 mg/litre.
- 25 8. A medium as claimed in claim 1 wherein the medium further comprises a lipid factor in an amount 0.05 - 10 mg/litre.
9. A medium as claimed in any of the preceding claims wherein the iron source is an inorganic ferric or ferrous salt present in an amount 0.25 - 5 mg/litre.
- 30 10. A medium as claimed in any of the preceding claims wherein the growth factor is selected from insulin, PDGF, thyroxine T₃, thrombin, interleukin, progesterone, hydrocortisone and vitamin E.
- 35 11. A medium as claimed in claim 10 wherein the growth factor is Nucellin.
12. A medium as claimed in any of the preceding claims containing a peptide digest, hydrolysate or extract.
13. A medium as claimed in any of the preceding claims which is essentially free from hypoxanthine and thymidine.
- 40 14. A medium as claimed in claim 13 containing methotrexate.
15. A CHO cell line adapted to grow in a culture medium as defined in any of claims 1-14.
- 45 16. A dhfr⁻ CHO cell line transfected with a gene encoding a biologically active protein and a dhfr selectable marker gene adapted to grow in a culture medium as defined in any of claims 1-14.
17. A dhfr⁻ CHO cell line as claimed in claim 16 wherein the biologically active protein is an antibody.
- 50 18. A dhfr⁻ CHO cell line claimed in claim 17 wherein the antibody is an anti-CDW52 antibody.

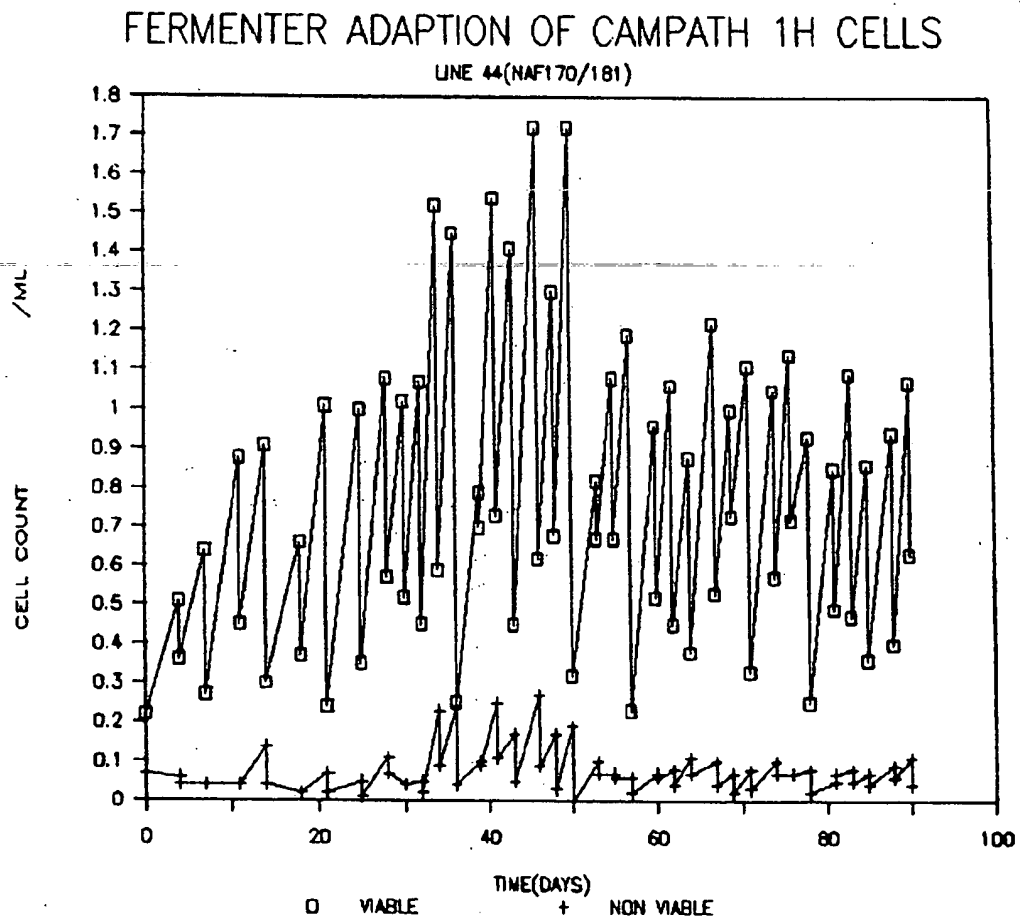


Figure 1.

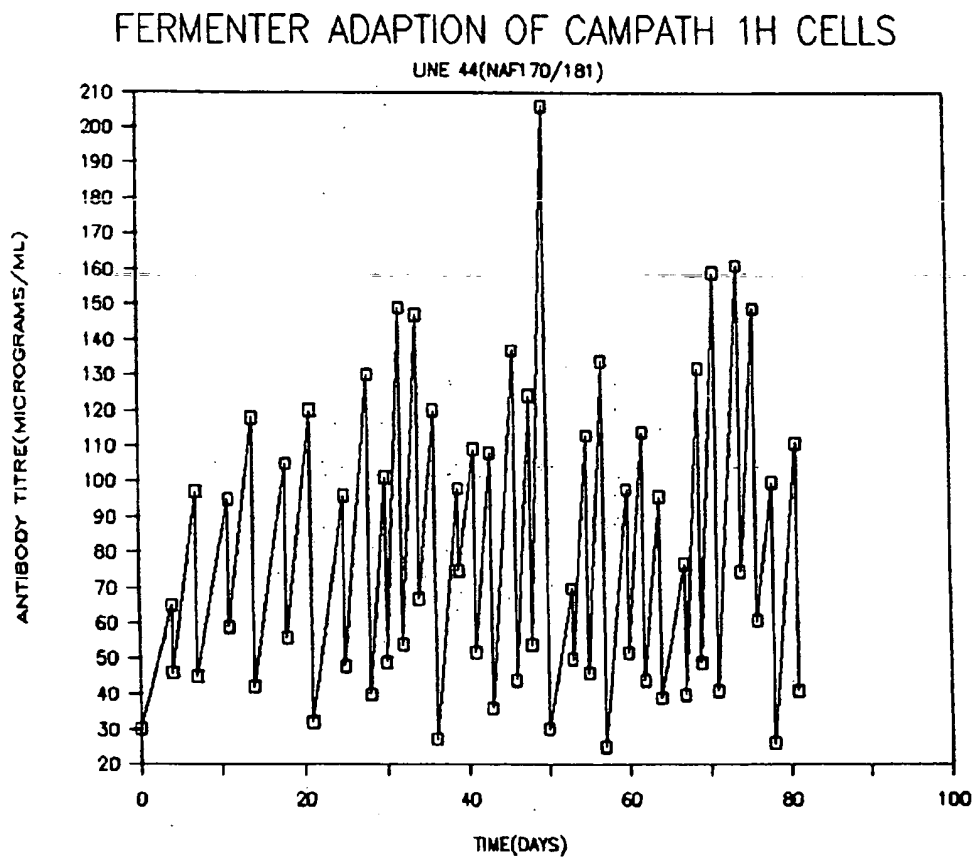


Figure 2.



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Culture medium for CHO-cells and adapted CHO-cells.

A biochemically defined culture medium for culturing engineered Chinese hamster ovary (CHO) cell lines, which is essentially free from protein, lipid and carbohydrate isolated from an animal source, comprising water, an osmolality regulator, a buffer, an energy source, amino acids including L-glutamine, an inorganic or recombinant iron source, and a synthetic or recombinant growth factor, and optionally non-ferrous metal ions vitamins and cofactors; also cells adapted to grow in such a culture medium.



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EUROPEAN SEARCH REPORT

Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP-A-0 325 190 (BOEHRINGER MANNHEIM GmbH) * The whole document *	1-14	C 12 N 5/02 C 12 N 5/10
X	EP-A-0 390 327 (ELI LILLY AND COMPANY) * Page 4, example 1 *	1-12	
X	US-A-4 767 704 (W.L. CLEVELAND) * The whole document *	1-12	
X	EP-A-0 389 786 (W.R. GRACE & CO.-CONN.) * Pages 11-13, table I *	1-12	
X	WO-A-8 800 967 (THE UNIVERSITY OF NEW SOUTH WALES) * Pages 5-13, examples 1-2; page 24, claim 7 *	1-9, 12	
X	CHEMICAL ABSTRACTS, vol. 110, no. 19, 8th May 1989, page 652, abstract no. 171821n, Columbus, Ohio, US; & JP-A-63 196 268 (KANEGAFUCHI CHEMICAL INDUSTRY CO., LTD) 14-08-1988 * Abstract *	1-12	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N C 07 K
X	CHEMICAL ABSTRACTS, vol. 108, no. 23, 6th June 1988, page 514, column 2, abstract 202886c, COLUMBUS, OHIO, US; V. FEYS et al.: "Expression of functional mouse antibodies directed against the tumor marker human placental alkaline phosphatase in non-lymphoid cells", & INT. J. CANCER, 1988 (Suppl. 2) 26-27 * Abstract *	15-17	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 07-04-1992	Examiner FERNANDEZ Y BRANAS F.J.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p>			

EP 0 FORM 1503 (04.92) (P0401)



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CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid,
namely claims:
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions,
namely:

see sheet -B-

- ☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid,
namely claims:
- ☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims,
namely claims:



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EUROPEAN SEARCH REPORT

Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claims	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	---	18	
Y,D	EP-A-0 328 404 (MEDICAL RESEARCH COUNCIL) * Whole document *	18	
X	---	15,16	
	JOURNAL OF BIOCHEMISTRY, vol. 106, 1989, pages 23-28, TOKYO, JP; M. TSUJIMOTO et al.: "Purification and characterization of recombinant human interleukin 5 expressed in Chinese Hamster ovary cells" * Whole article *		
X	---	15,16	
	BIOTECHNOLOGY, vol. 5, July 1987, pages 720-725, NEW YORK, US; G. ZETTLWEISS et al.: "Expression of biologically active human antitrombin III in Chinese Hamster ovary cells" * Whole article *		
X,P	---	15-18	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
	BIOTECHNOLOGY, vol. 9, January 1991, pages 64-68, NEW YORK, US; M.J. PAGE et al.: "High level expression of the humanized monoclonal antibody campath-1H in Chinese Hamster Ovary cells" * Whole article *		
A	---	1-14	
	R.I. FRESHNEY: "CULTURE OF ANIMAL CELLS", 1988, Alan R. Liss, New York, US; * Pages 71-84 *		
A	---		
	EP-A-0 316 068 (COLLABORATIVE RESEARCH, INC.) * Page 10, example 7 *		
		-/-	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 07-04-1992	Examiner FERNANDEZ Y BRANAS F.J.
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			

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EUROPEAN SEARCH REPORT

Page 3

Application Number

EP 91 30 9596

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 307 247 (GENENTECH INC.) * The whole document, especially examples 2,3 and 5 * -----	1-14	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 07-04-1992	Examiner FERNANDEZ Y BRANAS F.J.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>Y : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>Δ : member of the same patent family, corresponding document</p>			

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EP 91 30 9596 -B-

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims 1-14 : A biochemically defined culture medium as defined in claims 1 to 14.
2. Claims 15-18 : CHO cell line and genetically modified CHO cell lines as defined in claims 16 - 18.